HUMAN CERVICAL CANCER PROTOONCOGENE AND PROTEIN ENCODED THEREIN

Cross Reference to Related Application

This application is a continuation patent application of PCT Patent Application No. PCT/KR00/00284, which was filed on March 30, 2000, designating the United States of America.

Field of the Invention

The present invention relates to a novel protooncogene and protein encoded therein, and more particularly, to a human cervical cancer 1 protooncogene and a protein derived therefrom, which can be used in diagnosis of various cancers.

Background of the Invention

Higher animals including man each carry approximately 100,000 genes, but only about 15% thereof is expressed, and characteristics of individual's biological process, e.g., genesis, differentiation, homeostasis, responses to stimuli, control of cell segmentation, aging and apoptosis(programmed cell death), are determined depending on which genes are expressed(see Liang, P. and A. B. Pardee, *Science,* **257**: 967-971(1992)).

Pathogenic phenomena such as tumorigenesis are caused by gene mutation which brings about changes in the mode of gene expression. Therefore, comparative studies of gene expressions in various cells have

been conducted to provide bases for establishing viable approaches to the understanding of diverse biological phenomena.

For example, the mRNA differential display(DD) method suggested by Liang and Pardee is effective in elucidating the nature of tumor suppressor genes, cell cycle-related genes and transcriptional regulatory genes that control apoptosis(see Liang, P. and A. B. Pardee *supra*). Further, the DD method has been widely used in examining the interrelationship of various genes in a cell.

It has been reported that tumorigenesis is caused by various genetic changes such as the loss of chromosomal heterozygosity, activation of oncogenes and inactivation of tumor suppressor genes, e.g., p53 gene(see Bishop, J. M., *Cell,* **64**: 235-248(1991); and Hunter, T., *Cell,* **64:** 249-270(1991)). Further, it has been reported that 10 to 30% of human cancer arises from the activation of oncogene through amplification of protooncogenes.

Therefore, the activation of protooncogenes plays an important role in the etiology of many tumors and there has existed a need to identify protooncogenes.

The present inventor has endeavored to unravel examine the mechanism involved in the tumorigenesis of cervical cancer; and, has unexpectedly found that a novel protooncogene, human cervical cancer 1(HCCR-1), is specifically overexpressed in cancer cells. This protooncogene can be effectively used in diagnosis, prevention and treatment of various cancers, e.g., leukemia, lymphoma, kidney, liver, lung, ovary and uterine cervix cancers.

Summary of the Invention

Accordingly, the primary object of the present invention is to provide a novel protoncogene and a fragment thereof.

Other objects of the present invention are to provide:

a recombinant vector containing said protooncogene or a fragment thereof and a microorganism transformed therewith;

a protein encoded in said protooncogene and a fragment thereof;

a kit for diagnosis of cancer containing said protooncogene or a fragment thereof;

a kit for diagnosis of cancer containing said protein or a fragment thereof;

an anti-sense gene having a base sequence complementary to that of said protooncogene or a fragment thereof; and

a process for treating or preventing cancer by using said anti-sense gene.

In accordance with one aspect of the present invention, there is provided a novel protooncogene having the nucleotide sequence of SEQ ID No:1 or a fragment thereof.

In accordance with another aspect of the present invention, there is provided a recombinant vector containing said protooncogene or a fragment thereof and a microorganism transformed with said vector.

In accordance with still another aspect of the present invention, there is provided a protein having the amino acid sequence of SEQ ID No:2 or a fragment thereof derived from said protooncogene or a fragment thereof.

Brief Description of the Drawings

The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings which respectively show;

Fig. 1 : the DD identification of altered gene expression in normal cervix tissue, primary cervical cancer tissue, metastatic lymph node tissue and CUMC-6 cervical cancer cells.

Fig. 2 : the prediction of the hydrophobicity of transmembrane regions in the protooncogene of the present invention using TMPRED program.

Fig. 3 : the results of northern blot analyses for HCCR-1 gene expressed in normal cervical tissues, cervical cancer tissues and cervical cancer cell lines(CaSki and CUMC-6);

Fig. 4 : the results of northern blot analyses for HCCR-1 gene expressed in normal lung tissue and lung cancer cell lines (NCI-H358, NCI-H460, NCI-H441, NCI-H1299, NCI-H520, NCI-H2009, and NCI-H157);

Fig. 5A : the results of northern blot analyses for HCCR-1 gene expressed in normal human 12-lane multiple tissues;

Fig. 5B : the results obtained with the same sample of Fig. 5A hybridized with β-actin;

Fig. 6A : the results of northern blot analyses for HCCR-1 gene expressed in human cancer cell lines;

Fig. 6B : the results obtained with the same sample of Fig. 6A hybridized with β-actin;

Fig. 7A : the results of northern blot analyses for HCCR-1 gene expressed in human tumor tissues and their normal counterparts;

Fig. 7B : the results obtained with the same sample of Fig. 7A hybridized with β-actin;

Fig. 8 : a micrograph illustrating representative characteristics of *in situ* hybridized human cervical cancer tissues;

Fig. 9 : a phase-contrast feature of monolayer-cultured wild type NIH/3T3 cells;

Fig. 10 : a phase-contrast feature of monolayer-cultured HCCR-1 cells;

Fig. 11 : hematoxylin-eosin staining of monolayer-cultured HCCR-1 cells;

Fig. 12 : a transmission electron micrograph illustrating representative characteristics of cultured HCCR-1 cells;

Fig. 13 : tumorigenicity of HCCR-1 cells in nude mouse;

Fig. 14 : hematoxylin-eosin staining of subcutaneous tumour nodules taken from nude mice;

Fig. 15 : transmission electron micrographs illustrating representative characteristics of nude mice-derived subcutaneous tumor tissue;

Fig. 16 : phase-contrast features of monolayer-cultured nude mice-derived HCCR-1N cells;

Fig. 17 : sodium dodecyl sulfate (SDS)-PAGE results showing protein expression patterns before and after the IPTG induction;

Fig. 18 : the result of western blotting analysis of NIH/3T3 cells without transfection(wild type), NIH/3T3 transfected with pcDNA3 vector alone(pcDNA3) and HCCR-1 cells;

Fig. 19 : the result of western blotting analysis of human tumour tissues of kidney, lung, ovary and cervix and their normal counterparts;

Fig. 20 : the immunohistochemical study of HCCR-1-transfected

NIH/3T3 cells against reticulin fibers (x250);

Fig. 21 : the expression of epithelial marker, keratin in HCCR-1-transfected NIH/3T3 cells (x250);

Fig. 22 : the expression of epithelial membrane antigen in HCCR-1-transfected NIH/3T3 cells (x250);

Fig. 23 : the expression of mesenchymal marker, vimentin in HCCR-1-transfected NIH/3T3 cells (x250);

Fig. 24 : the PKC activities in NIH/3T3 cells without transfection(wild-type), NIH/3T3 transfected with pcDNA3 vector alone(pcDNA3) and NIH/3T3 transfected with HCCR-1 protooncogene (HCCR-1 cells);

Fig. 25 : the telomerase activities in 293 cells, +RNase, NIH/3T3 cells without transfection(wild-type), NIH/3T3 transfected with pcDNA3 vector alone(pcDNA3) and NIH/3T3 transfected with HCCR-1 protooncogene(HCCR-1 cells);

Fig. 26A : the results of RT-PCR amplification of HCCR-1 cDNA in H-358 lung carinoma cell lines treated with anti-sense oligodeoxynucleoties;

Fig. 26B : the results obtained with the same sample of Fig. 26A hybridized with β-actin;

Fig. 27 : growth curves of H-358 lung carcinoma cell lines treated with sense, missense or anti-sense HCCR-1 ODN, and untreated parental cells;

Fig. 28 : HCCR-1 protein expressions in fetal 16-(F16), 18-(F18), 20-(F20), postnatal 1-(P1), 7-(P7), 14-day(P14) and adult rat kidney tissue extracts;

Fig. 29 : immunohistochemical staining of 20 day-old fetal rat kidney (x42); and

Fig. 30 : differential-interference contrast microscopy of 18 day-old fetal rat kidney illustrating HCCR-1 immunostaining in the basolateral plasma membrane of medullary collecting duct (x220).

Detailed Description of the Invention

The novel protooncogene of the present invention, i.e., human cervical cancer 1(hereinafter "HCCR-1 protooncogene"), consists of 2118 base pairs and has the DNA sequence of SEQ ID NO:1.

In SEQ ID NO: 1, the full open reading frame corresponding to base Nos. 9 to 1088 is a protein encoding region and the predicted amino acid sequence derived therefrom is shown in SEQ ID NO: 2 which consists of 360 amino acids("HCCR-1 protein"). Further, the region corresponding to base Nos. 9 to 83 of SEQ ID NO: 1 encodes a signal peptide with the predicted amino acid sequence of amino acid Nos. 1 to 25 in SEQ ID NO: 2; and the region represented by nucleotide No. 435 to 494 of SEQ ID NO: 1 encodes a single transmembrane domain having the predicted amino acid sequence of amino acid Nos. 143 to 162 of SEQ ID NO: 2. This suggests that the protooncogene of the present invention is a membrane-bound gene.

A single potential N-glycosylation site(corresponding to base Nos. 945 to 953 of SEQ ID NO: 1 and amino acid Nos. 313 to 315 of SEQ ID NO: 2) is present at the C-terminal side of the HCCR-1 protein, which suggests that HCCR-1 is a type II membrane protein. The polyadenylation signal corresponds to the nucleotide Nos. 2008-2012 of SEQ ID NO:1.

The predicted extracellular domain of HCCR-1 corresponds to base Nos. 495-1088 with the predicted amino acid sequence of amino acid Nos. 163-360 consisting of 198 amino acids with 5 cysteine residues. The

predicted intracellular domain contains 117 amino acids(corresponding to nucleotide Nos. 84-434 of SEQ ID NO:1 and amino acid Nos. 26-142 of SEQ ID NO:2) with two potential protein kinase C(PKC) phosphorylation sites at Ser-42 and Ser-48, and two potential N-myristylation sites at Gly-34 and Gly-38. Further computer-assisted analyses indicate that HCCR-1 is markedly hydrophobic and possesses a characteristic single membrane-spanning domain and pre-secretory signal peptide as shown in Fig. 2.

In consideration of the degeneracies of codons and the preferred codons in a specific animal wherein the protooncogene of the present invention is to be expressed, various changes and modifications of the DNA sequences of SEQ ID NO:1 may be made, e.g., in the coding area thereof without adversely altering the amino acid sequence of the expressed protein, or in the non-coding area without adversely affecting the expression of the protooncogene. Therefore, the present invention also includes, in its scope, a polynucleotide having substantially the same base sequence as the inventive protooncogene, and a fragment thereof. As used herein, "substantially the same polynucleotide" refers to a polynuleotide whose base sequence shows 80% or more, preferably 90% or more, most preferably 95% or more homology to the protooncogene of the present invention.

The protein expressed from the protooncogene of the present invention consists of 360 amino acids and has the amino acid sequence of SEQ ID NO: 2. The molecular weight of this protein is about 40 kDa. However, various substitution, addition and/or deletion of the amino acid residues of protein may be performed without adversely affecting the protein's function. Further, a portion of the protein may be used when a specific purpose is to be fulfilled. These modified amino acids and fragments thereof are also included in the scope of the present invention.

Therefore, the present invention includes, in its scope, a polypeptide having substantially the same amino acid sequence as the protein derived from the oncogene of the present invention and a fragment thereof. As used herein, "substantially the same polypeptide" refers to a polypeptide whose amino acid sequence shows 80% or more, preferably 90% or more, most preferably 95% or more homology to the amino acid sequence of SEQ ID NO:2.

The protooncogene, or the protein, of the present invention can be obtained from human cancer tissues or synthesized using a conventional DNA or peptide synthesis method. Further, the gene thus prepared may be inserted to a conventional vector to obtain an expression vector, which may, in turn, be introduced into a suitable host, e.g., an *E. coli* or yeast cell, The cells transformed with a vector containing the HCCR-1 protooncogene or a fragment thereof is hereinafter referred to a "HCCR-1 cell".

The transformed host may then be used in producing the inventive DNA or protein on a large scale. For example, *E. coli* JM109 is transfected with HCCR-1 protooncogene by using pGEM-T easy vector and the JM109/HCCR-1 was deposited on October 11, 1999 with the Korean Collection for Type Cultures(KCTC)(Address: Korea Research Institute of Bioscience and Biotechnology(KRIBB), #52, Oun-dong, Yusong-ku, Taejon, 305-333, Republic of Korea) under the accession number, KCTC 0667BP, in accordance with the terms of Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

In preparing a vector, expression-control sequences, e.g., promoter. terminator, self replication sequence and secretion signal, are suitably selected depending on the host cell used.

The overexpression of the protooncogene of the present invention

occurs not in normal cervical and lung tissues but in cervical cancer tissues, cervical cancer cell lines and lung cancer cell lines. This suggests that the protooncogene of the present invention induces cervical and lung cancers. Further, when a normal fibroblast cell, e.g., NIH/3T3 cell line, is transfected with the protooncogene of the present invention, an abnormal cells is produced. Morphological characterizations with optical and electronic microscopes show that the abnormal cell has the form of a tumor cell.

When the normal fibroblast cell transfected with the protooncogene of the present invention is injected into the posterial lateral aspect of a nude mouse, tumorigenesis is observed after about 21 days from the injection, the tumor size becoming 1.5 cm x 1.5 cm in 40 days. By using hematoxylin-eosin dye method, it can be confirmed that the tumor cells are cancerous. The formation of the epithelial carcinoma can also be confirmed by using transmission electron microscopy and immunhistochemical staining methods.

In addition to epithelial tissues such as cervical and lung cancer tissues, the overexpression of the protooncogene of the present invention is also observed in various other cancer tumors such as leukemia, lymphoma, kidney, liver and ovarian cancers. Therefore, the protooncogene of the present invention is believed to be a factor common to all forms of various cancer and it can be advantageously used in the diagnosis of various cancers and the production of a transformed animal as well as in an anti-sense gene therapy.

A diagnostic method that can be performed using the protooncogene of the present invention may comprise, for example, the steps of hybridizing nucleic acids separated from the body fluid of a subject with a probe containing the protooncogene of the present invention or a fragment thereof, and determining whether the subject has the protooncogene by using a

conventional detection method in the art. The presence of the protooncogene may be easily detected by labeling the probe with a radioisotope or an enzyme. Therefore, a cancer diagnostic kit containing the protooncogene of the present invention or a fragment thereof is also included in the scope of the present invention.

A transformed animal produced by introducing the protooncogene of the present invention into a mammal, e.g., a rat, is also included in the scope of the present invention. In producing such a transformed animal, it is preferred to introduce the inventive protooncogene to a fertilized egg of an animal before the 8th cell cycle stage. The transformed animal can be advantageously used in screening for carcinogens or anticancer agents such as antioxidants.

The present invention also provides an anti-sense gene which is useful in a gene therapy. As used herein, the term "an anti-sense gene" means a polynucleotide comprising a base sequence which is fully or partially complementary to the sequence of the mRNA which is transcribed from the protooncogene having the base sequence of SEQ ID NO:1 or a fragment thereof, said nucleotide being capable of preventing the expression of the open reading frame(ORF) of the protooncogene by way of attaching itself to the protein-binding site of mRNA.

An example of the anti-sense gene of the present invention is a 18-mer HCCR-1 anti-sense oligodeoxinucleotide(ODN) having the base sequence of SEQ ID NO:3. Therefore, the present invention also includes, in its scope, a polynucleotide comprising substantially the same base sequence as SEQ ID NO:3 and a fragment thereof.

The present invention also includes within its scope a process for treating or preventing cancer in a subject by way of administering a

therapeutically effective amount of the inventive anti-sense gene thereto.

In the inventive anti-sense gene therapy, the anti-sense gene of the present invention is administered to a subject in a conventional manner to prevent the expression of the protooncogene. For example, the anti-sense ODN is mixed with a hydrophobized poly-L-lysine derivative by electrostatic interaction in accordance with the method disclosed by Kim, J.S. et al.(*J. Controlled Release*, **53**, 175-182(1998)) and the resulting mixed anti-sense ODN is administered intravenously to a subject.

The present invention also includes within its scope an anti-cancer composition comprising the anti-sense gene of the present invention as an active ingredient, in association with pharmaceutically acceptable carriers, excipients or other additives, if necessary. The pharmaceutical composition of the present invention is preferably formulated for administration by injection.

The amount of the anti-sense gene actually administered should be determined in light of various relevant factors including the condition to be treated, the chosen route of administration, the age and weight of the individual patient, and the severity of the patient's symptoms.

The protein expressed from the inventive protooncogene may be used in producing an antibody useful as a diagnostic tool. The antibody of the present invention may be prepared in the form of a monoclonal or polyclonal antibody in accordance with any of the methods well known in the art by using a protein having the amino acid sequence of SEQ ID NO:2 or a fragment thereof. Cancer diagnosis may be carried out using any of the methods known in the art, e.g., enzyme linked immunosorbentassay(ELISA), radioimmunoassay(RIA), sandwich assay, western blot or immunoassay blot on polyacrylic gel, to asses whether the protein is expressed in the body fluid

of the subject. Therefore, a cancer diagnostic kit containing the protein having the amino acid sequence of SEQ ID NO:2 or a fragment thereof is also included in the scope of the present invention.

A continuously viable cancer cell line may be established by using the protooncogene of the present invention, and such a cell line may be obtained, for example, from tumor tissues formed on the back of a nude mouse by injecting fibroblast cells transformed with the protooncogene of the present invention. The cell lines thus prepared may be advantageously used in searching for anti-cancer agents.

The following Examples and Test Examples are given for the purpose of illustration only, and are not intended to limit the scope of the invention.

Example 1 : Cultivation of tumor cells and separation of total RNA

Step 1-1 : Cultivation of tumor cells

For differential display of mRNA, normal cervical tissues, untreated primary cervical cancer tissues and metastatic common iliac lymph node tissues were obtained from cervical cancer patients who underwent radical hysterectomy. The human cervical cancer cell line used in the differential display method was CUMC-6 cell line described by Kim *et al.*,(*Gynecol. Oncol.*, 62: 230-240(1996)).

Cells from the above-described tissues and CUMC-6 were maintained on Waymouth's MB 752/1 medium (Gibco) supplemented with 2 mmol/L of glutamine, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 10% of fetal bovine serum (Gibco). Only the cell suspensions with greater than 95% viability, as assessed by trypan blue dye exclusion described by Freshney("Culture of Animal Cells: A Manual of Basic

Technique" 2nd Ed., A. R. Liss, New York(1987)) were used in the present experiments.

Step 1-2 : Isolation of total RNA and differential display of mRNA

Total RNAs were extracted from normal cervical tissues, primary cervical cancer tissues, metastatic common iliac lymph node tissues and CUMC-6 cells obtained in Step 1-1 using a commercial system (RNeasy total RNA kit) provided by Qiagen (Qiagen Inc., Germany) and the removal of DNA contaminants from the RNAs was accomplished using Message clean kit (GenHunter Corp., Brookline, MA).

Example 2 : Differential display reverse transcription(DDRT)-PCR

Differential display reverse transcription was performed in accordance with the reverse transcription-polymerase chain reaction (RT-PCR) method described by Liang and Pardee(1992), *supra*, with minor modifications.

First, reverse transcription was carried out using 0.2 μg each of the total RNAs obtained in Step 1-2 of Example 1 and one of the three primers, i.e., H-T11G, H-T11C, or H-T11A, as anchored oligo-dT primers (RNAimage kit, GenHunter Cor., MA, USA).

Then PCR was conducted using the same anchored primers and one of the arbitrary 5' 13 mer (RNAimage primer sets 1-4, H-AP 1-32) in the presence of 0.5 mM [α-^{35}S]-labeled dATP (1200 Ci/mmol). The PCR thermal cycle was repeated 40 times, the cycle being composed of: 95 ℃ for 40 sec., 40 ℃ for 2 min. and 72 ℃ for 40 sec., and finally the reaction was carried out at 72 ℃ for 5 min.

PCR-amplified fragments were resolved in 6% polyacrylamide sequencing gels. Differentially expressed fragments were identified by inspection of autoradiograms.

Bands of more than 200 base pairs, CC214, were excised from the dried sequencing gel. The CC214 cDNAs were eluted by boiling for 15 min and reamplified with the same primer pairs and PCR conditions as used in the above amplification step except that no [α -^{35}S]-labeled dATP and 20 μM dNTPs were used.

Example 3 : Cloning

The reamplified CC214 PCR product obtained as above was inserted into the pGEM-T Easy vector using an TA cloning system (Promega, USA) in accordance with the manufacturer's instructions.

Step 3-1 : Ligation

2 μl of the reamplified CC214 PCR product obtained in Example 2, 1 μl of pGEM-T easy vector (50 ng), 1 μl of T4 DNA ligase 10X buffer solution and 1 μl of T4 DNA ligase(3 weiss units/μl; T4 ligase, Promega, USA) were charged into a 0.5ml tube and distilled water was added thereto to a final volume of 10 μl. The ligation reaction mixture was incubated overnight at 14℃.

Step 3-2 : TA cloning transformation

TA cloning transformation was performed using the following protocol.

E. coli JM109(Promega, WI, USA) was incubated in 10 ml of LB

broth(Bacto-trypton 10g, Bacto-yeast extract 5g, NaCl 5g) until the optical density at 600nm reached about 0.3 to 0.6. The cultured mixture was kept at 0℃ for 10 minutes and centrifuged at 4000 rpm at 4℃ for 10 minutes. The supernatant was removed and cells were harvested. The harvested cell pellet was exposed to 10ml of 0.1M $CaCl_2$ at 0℃ for 30 minutes to 1 hour to obtain competent cells. The resultant was centrifuged at 4000 rpm at 4℃ for another 10 minutes and the collected cells were suspended in 2ml of 0.1M $CaCl_2$ at 0℃.

200 μl of the competent cell suspension was transferred to a microfuge tube and 2 μl of the ligation product obtained in Step 3-1 was added thereto. The mixture was incubated in a water bath at 42 ℃ for 90 seconds and rapidly cooled to 0℃. Added thereto was 800 μl of SOC medium (Bacto-trypton 2.0g, Bacto-yeast extract 0.5 g, 1M NaCl 1 ml, 1M KCl 0.25 ml, TDW 97 ml, 2M Mg^{2+} 1 ml, 2M glucose 1ml) and the mixture was incubated at 37℃ for 45 minutes at 220 rpm in a rotary shaking incubator.

LB agar plates containing ampicillin(50ul/ml) were prepared by spreading 25μl of X-gal (40mg/ml stock in dimethylformamide) on top of agar with a glass spreader. 25μl of the transformed cells thus obtained was spread thereon and the plates were incubated at a 37℃ incubator overnight. White colonies were loaded on an LB agar plate containing ampicillin and transformed *E. coli*, i.e., JM109/CC214 were selected and incubated in a terrific broth(TDW 900 ml, Bacto-trypton 12 g, Bacto-yeast extract 24 g, glycerol 4 ml, 0.17M KH_2PO_4, 0.72 N K_2HPO_4 100 ml).

Example 4 : Separation of recombinant plasmid DNA

The CC214 DNA of the transformed *E. coli* was separated by employing Wizard™ Plus Minipreps DNA Purification Kit(Promega, USA) in accordance with the manufacturer's instructions.

A portion of the plasmid DNA thus separated was treated with ECoRI enzyme and subjected to gel electrophoresis to confirm the insertion of CC214 partial sequence in the plasmid.

Example 5 : Sequence Analysis of DNA

The CC214 PCR product obtained in Example 2 was subjected to PCR in accordance with the conventional method and the cloned, reamplified CC214 PCR fragments were subjected to sequence analysis according to the dideoxy chain termination method using a Sequenase version 2.0 DNA sequencing kit (United states Biochemical, Cleveland, OH) in accordance with the manufacturer's instructions.

The base sequence of the DNA corresponds to nucleotide Nos. 1883-2088 in SEQ ID NO:1 and is designated "CC214".

The differential display reverse transcription polymerase chain reaction(DDRT-PCR) of the 206 bp cDNA fragment, i.e., CC214 obtained above was carried out using a 5' arbitrary primer H-AP21 and a 3' H-T11C anchored primer and resolved by electrophoresis. Identification of altered gene expression by DD in the primary cervical cancer, metastatic lymph node tissue and CUMC-6 cells is shown in Fig 1. As can be seen in Fig. 1, the 206 bp cDNA fragment, i.e., CC214 was expressed in the cervical cancer, metastatic tissue and CUMC-6 cervical cancer cells but not in the normal

tissue.

Example 6 : Full length cDNA sequence analysis of the HCCR-1 protooncogene

A bacteriophage λ gt11 human lung embryonic fibroblast cDNA library (see Miki, T. et al., Gene, 83:137-146(1989)) was screened by plaque hybridization with ^{32}P-labeled CC214 as a probe. The full-length HCCR-1 cDNA clone, containing a 2118 bp insert in pCEV-LAC vector was obtained from the human lung embryonic fibroblast cDNA library and registered at GenBank on November 5, 1999 under the accession number AF195651.

HCCR-1 clone inserted into λpCEV vector(see Miki, T. et al., supra) was excised out of the phage in the form of the ampicilline-resistant pCEV-LAC phagemid vector(see Miki, T. et al., supra) by *Not* I cleavage.

To make a HCCR-1 plasmid DNA, pCEV-LAC vector containing HCCR-1 gene was ligated with T4 DNA ligase and ligated clone was transformed into *E. coli* JM109.

The transformed *E. coli* JM109/HCCR1 thus obtained was deposited on October 11, 1999 with the Korean Collection for Type Cultures(KCTC)(Address: Korea Research Institute of Bioscience and Biotechnology(KRIBB), #52, Oun-dong, Yusong-ku, Taejon, 305-333, Republic of Korea) under the accession number, KCTC 0667BP, in accordance with the terms of Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure.

The full sequence of HCCR-1 consists of 2118 bp which is identified in SEQ ID NO:1.

In SEQ ID NO:1, the full open reading frame of the HCCR-1 protooncogene of the present invention corresponds to nucleotides No. 9 to 1088 and is predicted to encode amino acid sequence shown in SEQ ID NO:2 which consists of 360 amino acids. Further, the region corresponding to nucleotide Nos. 9 to 83 of SEQ ID NO:1 encodes a signal peptide having 25 amino acids corresponding to amino acid Nos. 1 to 25 of SEQ ID NO:2; the region of nucleotide Nos. 435 to 494 of SEQ ID NO:1 encodes a single transmembrane domain whose amino acid sequence corresponds to amino acid Nos. 143 to 162 in SEQ ID NO:2. This indicates that the protooncogene of the present invention is a membrane-bound gene.

A single potential N-glycosylation site(corresponding to base Nos. 945 to 953 of SEQ ID NO: 1 and amino acid Nos. 313 to 315 of SEQ IS NO: 2) is present at the C-terminal side of the HCCR-1 protein, which suggests that HCCR-1 is a type II membrane protein. The polyadenylation signal corresponds to the nucleotide Nos. 2008-2012 of SEQ ID NO:1.

The predicted extracellular domain contains 198 amino acids with 5 cysteine residues. The predicted intracellular domain contains 117 amino acids(corresponding to nucleotide Nos. 84-434 of SEQ ID NO:1 and amino acid Nos. 26-142 of SEQ ID NO:2) with two potential PKC phosphorylation sites at Ser-42 and Ser-48, and two potential N-myristylation sites at Gly-34 and Gly-38. Further computer-assisted analyses indicated that HCCR-1 is markedly hydrophobic and possesses a characteristic single membrane-spanning domain and pre-secretory signal peptide as shown in Fig. 2. In Fig. 2, the X-axis represents the amino acid sequence number of the peptide of the present invention and the Y-axis, the hydrophobicity of the peptide.

Example 7 : Northern blot analysis of the HCCR-1 gene in various cells

Total RNAs were extracted from various tissues and cell lines as in Example 1.

To determine the level of HCCR-1 gene expression, 20 μg denatured total RNAs from each tissue or cell lines were electrophoresed through 1% formaldehyde agarose gel and transferred to nylon membranes (Boehringer-Mannheim, Germany). The blots were hybridized with a ^{32}P-labeled random-primed HCCR-1 full cDNA probe which was prepared using a rediprime II random prime labeling system(Amersham, England). The northern blot analysis was repeated twice and the results were quantified by densitometry and normalized with β -actin.

Fig. 3 shows the results of northern blot analyses for HCCR-1 gene expressed in normal cervical tissues, cervical cancer tissues and cervical cancer cell lines(CaSki and CUMC-6). As can be seen in Fig. 3, the transcription level of HCCR-1 is high in the cervical cancer tissues and cancer cell lines (CaSki(ATCC CRL 1550) and CUMC-6), but very low or undetactable in the normal cervical tissues.

Fig. 4 shows the results of northern blot analyses for HCCR-1 gene expressed in normal lung tissues and seven lung cancer cell lines, i.e., H358(ATCC NCI-H358), H460(ATCC NCI-H460), H441(ATCC NCI-H441), H299(ATCC NCI-H299), H520(ATCC NCI-H520), H2009(ATCC NCI-H2009), and H157(ATCC NCI -H157). As shown in Fig. 4, HCCR-1 transcription level in high level in lung cancer cell lines H358, H460, H1299, H520, and H157, but not detectable in the normal lung tissues.

Fig. 5A shows the results of northern blot analyses for HCCR-1 gene expressed in normal human 12-lane multiple tissues; brain, heart, skeletal

muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, lung and leukocyte tissues(Clontech). Fig. 5B shows the results obtained with the same samples hybridized with a β -actin probe to confirm mRNA integrity. As can be seen in Fig. 5A, HCCR-1 mRNA (~2.1 kb) is weakly present or absent in many normal tissues, but the level of expression was high in normal kidney tissue.

Fig. 6A shows the results of northern blot analyses for HCCR-1 gene expressed in human cancer cell lines; HL-60, HeLa, K-562, MOLT-4, Raji, SW480, A549 and G361(Clontech). Fog. 6B shows the results obtained with the same samples hybridized with a β-actin probe to confirm mRNA integrity. As can be seen in Fig. 6A, HCCR-1 is transcribed at a high level in the human leukaemia and lymphoma cell lines such as chronic myelogenous leukaemia K-562, Burkitt's lymphoma Raji, lymphoblastic leukaemia MOLT-4 and promyelocytic leukaemia HL-60 as well as HeLa cells.

K-562, MOLT-4 and HL-60, in particular, show higher transcription levels as compared with normal leukocyte by factors of approximately 190, 90 and 70, respectively. HCCR-1 expression levels in colorectal cancer SW480, lung cancer A549 and melanoma G361 cell lines are lower than those of leukemia and lymphoma.

Further, northern blotting analyses of the human kidney, liver, lung, ovary and cervix tumor tissues and their normal counterparts were carried out. As shown in Fig. 7A, HCCR-1 was transcribed at a high level in the human cancer cells, while the expression of HCCR-1 gene is barely observable in the normal cells. Fig. 7B shows the results obtained with the same samples hybridized with β-actin probe to confirm mRNA ingegrity.

Example 8 : Micrograph of *in situ* hybridized Human Cervical Cancer Tissues

For *in situ* hybridization, human cervical cancer tissue was fixed in periodate-lysine-paraformaldehyde, embedded in a wax according to the procedure described by Ahn et al.(*Am. J. Physiol.* **265**, F792 - F801 (1993)) and sectioned(5 μm). A full-length HCCR-1 cDNA fragment was used to synthesize a digoxigenin-labelled RNA probe. RNA *in situ* hybridization was carried out with the anti-sense RNA probe which was prepared using a DIG RNA Labelling Kit(Boehringer Mannheim). The sense RNA probe was used as a negative control.

Fig. 8 shows a micrograph illustrating representative characteristics of *in situ* hybridized human cervical cancer tissues. As can be seen in Fig. 8, the *in situ* hybridized cervical cancer tissues are confirmed to contain a high-level of HCCR-1 gene. No staining was detected in the surrounding normal fibrous tissues.

Example 9: Construction of expression vectors and transformation of cells

Step 9-1 : Preparation of a vector containing HCCR-1

An expression vector containing the coding region of HCCR-1 was constructed as follows.

First, the entire HCCR-1 cDNA obtained in Example 6 was inserted into the *Sal*I restriction site of a prokaryotic expression vector, pCEV-LAC(see Miki, T. *et al.*, *Gene,* 83: 137-146 (1989)). Then, the *Sal*I fragment was isolated from the pCEV-LAC/HCCR-1 vector.

Then, pcDNA3 (Invitrogen) was digested with *Xho*I to make a

compatible end with *Sal*I. The *Sal*I fragment containing the full length HCCR-1 coding sequence was inserted into the *Xho*I-digested pcDNA3. Lipofectamine (Gibco BRL) was used to introduce the resulting pcDNA3/HCCR-1 expression vector into NIH/3T3 cells(ACTC CRL, 1658, USA), followed by selection in a medium supplemented with G418 (Gibco). The resulting NIH/3T3 cells transfected with HCCR-1 was designated "HCCR-1 cells". Another population of NIH/3T3 cells containing pcDNA3 alone was prepared as a control and designated "pcDNA3 cells".

Step 9-2 : NIH/3T3 fibroblast cells transfected with the HCCR-1 protooncogene

The wild type normal NIH/3T3 cell, a differentiated fibroblast cell line, is a spindle shaped cell having a long slender nucleus and a scanty amount of cytoplasm as shown in Fig. 9. When HCCR-1 was expressed in the NIH/3T3 expressing HCCR-1(HCCR-1 cells) obtained in Step 9-1, the cell shape changes into a polygonal form with an ovoid nucleus and plump cytoplasm, as shown in Fig. 10.

Monolayer cultured HCCR-1-transfected NIH/3T3 cells which is stained with hematoxylin-eosin, exhibit nuclear pleiomorphism, distinct nucleoli, granular chromatin patterns, tumor giant cells and atypical mitotic figures as shown in Fig. 11.

For transmission electron microscopy(TEM), the cells and tissues were fixed with 2.5% glutaraldehyde in a phosphate buffer (pH 7.4). They were then postfixed with a 2% osmium tetroxide. Specimens were dehydrated in a graded series of ethanols and embedded in Epon 812. Ultrathin sections thereof were stained with uranyl acetate and lead citrate, and photographed by TEM(JEOL 1,200 EX, Tokyo, Japan).

The TEM picture shown in Fig. 12 reveals that cultured tumour cells have microvilli and well-developed organelles (inset). As can be seen in Fig. 12, the HCCR-1 cell has microvilli on the cell surface, lobulated nucleus with prominent nucleoli and well-developed rough endoplasmic reticula(rER) and Golgi complexes (circle). In Fig. 12, the scale bar corresponds to 3 μm. In higher magnification of the area indicated by circle (inset), the scale bar corresponds to 1 μm.

Example 10: Tumorigenicity and metastasis of HCCR-1 protooncogene in animal

To analyze tumourigenicity, 5 X 10^6 HCCR-1 cells were injected subcutaneously into the posterior lateral aspect of the trunk of 9 mice (5-week-old athymic nu/nu on BALB/c background). Nude mice were sacrificed when the subcutaneous tumors reached 1.5-2.5 cm in diameter.

All 9 mice injected with HCCR-1 cells showed palpable tumors after 21 days as shown in Fig 13.

Nude mice bearing HCCR-1 allografts display characteristics of an epithelial carcinoma. Fig. 14 shows hematoxylin-eosin staining of subcutaneous tumor nodules taken from the nude mice. The sections of the tumour nodules revealed typical epithelial cell nests separated by fibrous stroma.

Example 11 : Electron microscopy of HCCR-1 protooncogene - induced tumor tissue and establishment of new cancer cell line

Tumor tissues taken from the tumor nodules formed on the nude

mouse of Example 10 were examined with an electron microscope, which revealed that tumor nodules showed well-developed organelles and tumour cells are connected by desmosomes(Fig. 15). As shown in Fig. 15, the tumor tissue consists of tightly adhered cells with intercellular junction (circle). In Fig. 15, the scale bar corresponds to 3 μm. In higher magnification of the area indicated by circle illustrating desmosome (inset), the scale bar corresponds to 0.5 μm.

The cells obtained from the above tumour tissue was cultured in a conventional manner using 20% fetal bovine serum and the cultured cells were designated HCCR-1N cells which have cytological features similar to HCCR-1 cells *in vitro* as shown in Fig. 16.

Example 12: Determination of size of protein expressed after the transfection of *E. coli* with HCCR-1 protooncogene

A portion of HCCR-1 protooncogene corresponding to nucleotide Nos. 123-473 and predicted amino acid Nos. 39-155 was inserted into the multiple cloning site of pET-32b(+) vector(Novagen) and the resulting pET-32b(+)/HCCR-1 vector was transfected into *E. coli* BL21(ATCC 47092). The transfected *E. coli* was incubated using an LB broth medium in a rotary shaking incubator, diluted by 1/100, and incubated for 3 hours. 1mM isopropyl β-D-thiogalacto-pyranoside(IPTG, Sigma) was added thereto to induce the protein synthesis.

The *E. coli* cells in the culture were disrupted by sonication and subjected to gel electrophoresis using 12% sodium dodecyl sulfate(SDS) before and after the IPTG induction. Fig. 17 shows the SDS-PAGE results which exhibit a protein expression pattern of the *E. coli* BL21 strain

transfected with pET-32b(+)/HCCR-1 vector. After the IPTG induction, a significant protein band was observed at about 35kDa. This 35kDa fused protein contained an about 20kDa Trix·Tag thioredoxin protein expressed a the gene in pET-32b(+) vector.

Example 13: Production of antibody

The 35 kDa fused protein isolated from the *E. coli* BL21 strain transfected with pET-32b(+)/HCCR-1 vector in Example 12 was purified by using a His-Bind Kit (Novagen). Immunoblotting of the purified peptide confirmed the presence of a major amount of a 35kDa protein.

Then, two 6-seek old Sprague-Dawley rats each weighing about 150 g were each subcutaneously immunized with 1 mg of the peptide thus obtained, weekly for 3 times. Blood samples were obtained from the immunized rats and centrifuged to obtain a polyclonal serum. The anti-HCCR-1 activity of the polyclonal serum was determined and confirmed by enzyme-linked immunosorbent assay(1:10,000)

Example 14 : Immunoblot confirming Antibody specificity

For western blot analysis, those cells identified in Figs. 18 and 19 were harvested and lysed in a Laemmli sample buffer in accordance with the method described by Laemmli(*Nature* **227:** 680-685 (1970)). The cellular proteins were separated by 10% SDS-PAGE and then electroblotted onto nitrocellulose membranes. The membranes were incubated with the rat polyclonal anti-HCCR-1 serum prepared in Example 13 for 16 h. After washing, the membranes were incubated with a blocking solution containing

1:1,000 dilution of peroxidase-conjugated goat anti-rat immunoglobulin (Jackson ImmunoResearch) as a secondary antibody. Proteins were revealed by an ECL-Western blot detection kit (Amersham).

As shown in Fig. 18, HCCR-1 protein is overexpressed in HCCR-1 cells, while only faint bands are observed for the wild type and cells transfected with the vector alone(pcDNA3). This result illustrates the specificity of the anti-HCCR-1 antibodies in the polyclonal serum.

Further, the HCCR-1 antibody in the polyclonal serum recognized approximately 40 kDa protein in human protein extracts from different tissues. As shown in Fig. 19, human tumor tissues including carcinomas of the kidney, lung, ovary and cervix showed increased HCCR-1 protein expression when compared with their normal counterparts.

Example 15: Immunohistochemistry

The tumor nodules formed on the nude mouse of Example 9 were incubated with anti-vimentin, anti-keratin, anti-EMA(epithelial membrane antigen) antibodies (DAKO) and polyclonal antibody raised against HCCR-1, respectively. Then, immunohistochemistry was carried out on 5 μm-cryosections of the incubated tumor nodules.

Binding of primary antibody was visualized by biotinylated secondary antibody, avidin, biotinylated horseradish peroxidase and AEC(Aminoethyl Carbaxzole Substrate Kit) as the chromogen(HISTOSTAIN-BULK KITS, Zymed). The immunohistochemical study revealed that HCCR-1 transfection into NIH/3T3 cells caused the conversion of the cell nature from mesenchymal to epithelial. The cell nests were enveloped by reticulin fibers as shown in Fig.

20.

The cells showed coexpression of epithelial markers, such as keratin(Fig. 21) and epithelial membrane antigen (Fig. 22) and of the mesenchymal marker, vimentin(Fig. 23).

Example 16: Protein kinase C and telomerase activity assays

To ensure that HCCR-1 modulates the protein kinase C(PKC) activity in cells, PKC assay was performed using wild-type NIH/3T3 cells, pcDNA3-containing NIH/3T3 cells and HCCR-1-transfected NIH/3T3 cells prepared in Step 9-1 of Example 9.

PKC activity was measured using the SignaTECT™ Protein Kinase C Assay System (Promega) according to the manufacturer's instructions. PKC activity was defined as the difference of the amounts of PKC incorporated into substrate per minute in the absence and presence of phospholipids. Each value is the means ± s.d. of three independent experiments.

The result in Fig. 24 shows that the PKC activity of HCCR-1-transfected NIH/3T3 cells is about 10-fold higher than the wild-type.

To explain the tumorigenesis of HCCR-1, telomerase activities in wild-type NIH/3T3 cells, pcDNA3-containing NIH/3T3 cells and HCCR-1-transfected NIH/3T3 cells prepared in Step 9-1 of Example 9 were measured using the telomerase PCR-ELISA kit (Boehringer Mannheim) according to the manufacturer's instructions. Human telomerase-positive immortalized human kidney cells (293 cells) provided in the kit were used as a positive control.

Used as a negative control was the 293 cells pretreated with RNase

(+RNase). Assays were performed with an extract amount equivalent to 1 x 10^3 cells.

Results in Fig. 25 show the average mean optical density(OD) values from four separate experiments (means ± s.d.). Consistent with the previous study (Holt, S. E., Wright, W. E. and Shay J. W. *Mol. Cell Biol.* **16**, 2932-2939 (1996), wild-type NIH/3T3 cells showed detectable telomerase activity. HCCR-1 gene transfection raised the telomerase activity by a factor of about 7 as compared with the wild-type cells. The high telomerase activity of the 293 cells was nullified by pretreatment with RNase.

Example 17: Cell cycle experiments

Wild-type and HCCR-1-transfected NIH/3T3 cells cultured in a DMEM medium at mid-log phase were growth arrested by incubation in a DMEM medium containing 0.5% bovine calf serum for 36 h. Cells to be analyzed for the DNA content were harvested following trypsinization, and fixed in 70% ethanol. Fixed cells were then stained with propidium iodide as described by Hedley(*Flow Cytometry, DNA Analysis from Paraffin-embedded Blocks*; Darzynkiewicz, Z. & Crissman, H. A. eds., Academic Press, San Diego, 1990).

First, 50 μg/ml of a propidium iodide staining solution (Sigma) and 100 units per ml of RNase A (Boerhinger Mannheim) were added to 2 × 10^6 cells. After incubation for 1 h, the cellular DNA content was determined by fluorescence analysis at 488 nm using a FACS Caliber (Becton Dickinson). A minimum of 1 × 10^4 cells per sample was analyzed with Modfit 5.2 software.

In order to study whether there was an alteration in the growth

properties of HCCR-1-transfected NIH/3T3 cells, cell cycle profiles were examined. The cell contents of the wild type NIH/3T3 cells and HCCR-1 transfected NIH/3T3 cells(mid-log phase) in G_0/G_1, S, G_2/M phases were measured and the results are shown in Table I.

Table I

	Wild Type			HCCR-1 Cell		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Cell Content(%)	55.7	20.6	24	46.6	31.5	22.4

As can be seen from Table I, the percentage of wild-type and HCCR-1 transfected NIH/3T3 cells in the S-phase was 20.6% and 31.5%, respectively (mid-log phase). These results suggest that there was a signigicant shift of the cell pipulation out of the G_0/G_1-phase into the S-phase in HCCR-1 transfected NIH/3T3 cells.

To assess the serum-dependent cell cycle progression, cells were cultured in 0.5% bovine calf serum for 36 h. After incubation, cells were released with 20% bovine calf serum and harvested at indicated times. The cell contents of wild type NIH/3T3 cells and HCCR-1 transfected NIH/3T3 cells in G_0/G_1, S, G_2/M phases at indicated times were measured and the results are shown in Table II.

Table II

Time(h)	Cell Content(%)					
	Wild Type			HCCR-1 Cell		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
0	77	8.0	14.9	70	21.8	8.7
12	72.2	14	14.2	66.9	24.0	9.6
24	49.6	13.4	37.2	56.7	24.7	19.2
48	58.3	18.3	23.7	52.7	30.4	17.5

As can be seen from Table II, few cells remained in the S-phase in wild-type cells measured at 0 h (8%). In contrast, a considerable number of HCCR-1 cells measured at 0 h were still in the S-phase (21.8%), suggesting that constitutive overexpression of HCCR-1 allowed for a relative amount of resistance to serum deprivation-induced G_0/G_1 arrest. Following the release of cells from the growth arrest caused by serum-deprivation, there were consistent increases of over 10% in the S-phase populations of HCCR-1 cells as compared to wild-type cells at measured time intervals (12 h, 24 h and 48 h). Therefore, overexpression of HCCR-1 could deregulate cell growth by shortening the G_0/G_1-phase and increasing the S-phase population of cells.

Example 18: Construction of anti-sense oligodeoxinucleotide

Anti-sense and sense phosphorothioate oligodeoxynucleotide(ODNs) targeting the translation starting site of HCCR-1 mRNA were synthesized based on the human HCCR-1 cDNA sequence (GenBank accession number AF195651) by cyanoethylphosphoramidite chemistry on an automated DNA synthesizer (Expedite Nucleic Acid System, Framingham, MA).

The sequence of 18-mer HCCR-1 anti-sense ODN was 5'-CCTGGACATTTTGTCACC-3' (SEQ ID NO: 3; corresponding to nucleotide Nos. 66 to 83 of SEQ ID NO:1). The corresponding sense sequence, 5'-GGTGACAAAATGTCCAGG-3'(SEQ ID NO: 4), and missense sequence, 5'-CGCGGATATTTCCTCACC-3'(SEQ ID NO: 5) were used as controls.

Example 19: Cancer gene therapy using HCCR-1 antisense ODN

Step 19-1 : Inhibition of gene expression

Exponentially growing 2 X 10^5 H-358 lung carcinoma cells (ATCC CRL-5807) were detached by trypsin-EDTA and seeded in a 24-well plate. Lipofectamine (Gibco BRL) was used for oligodeoxynucleotide(ODN) treatment. Lipofectamine (5 μl/ml medium) was incubated with an appropriate amount of ODN to achieve a final concentration of 100 nM ODN, in the cell suspension for 30 minutes at room temperature. Then 1,000μl portions of the mixture were added directly to the cells on the 24-well plates and incubated for 1, 2, 3, 5 and 7 days, respectively. There was no cytotoxicity of the transfection reagent as controlled by trypan blue dye exclusion assay.

To observe the inhibitory effect of HCCR-1 anti-sense ODN, the cultured lung carcinoma cells were treated with 100 nM each of sense, missense and anti-sense ODNs obtained in Example 18, respectively. Inhibition of HCCR-1 expression in anti-sense ODN-treated lung carcinoma cells was demonstrated by reverse transcription-polymerase chain reaction (RT-PCR). The sequences of oligonucleotide primers used for RT-PCR, synthesized according to the coding region of HCCR-1 cDNA were as

follows: forward, 5'-GGGAGATGGAGCATTTGAGA-3' (SEQ ID NO:6, corresponding to nucleotides Nos. 376-395 of SEQ ID NO:1) and reverse, 5'-GCTTCCGGAAAGCATGATAG-3'(SEQ ID NO:7, corresponding to nucleotides 554-573 of SEQ ID NO:1.

The dose of anti-sense exerting inhibitory effect was related to the levels of HCCR-1 mRNA expression(Fig. 26A). 497 bp β-actin was used as an internal control to confirm mRNA integrity(Fig. 26B). A negative control(N) contained nuclease-free water instead of RNA template. 1000-bp ladder DNA size marker(M) was also used. As shown in Fig. 26A, the level of 198 bp HCCR-1 RT-PCR product decreased in a time-dependent manner by anti-sense ODN treatment. HCCR-1 gene expression was completely inhibited in cells treated with 100 nM of anti-sense HCCR-1 ODN for 7 days. In contrast, expected 198 bp HCCR-1 RT-PCR product was detected in cells treated with 100 nM of sense or missense HCCR-1 ODN, respectively, and in the untreated parental cells.

These results show that the treatment with 100 nM of anti-sense HCCR-1 ODN completely blocks HCCR-1 gene expressions in lung carcinoma cells.

Step 19-2 : Inhibition of cell growth

The growth phenotype of H-358 lung cancer cells treated with 100 nM of sense, anti-sense or missense HCCR-1 oligodeoxynucleotide was assessed by growth curve.

In three independent experiments, H-358 lung cancer cells were trypisinized and plated in the presence of 100 nM of sense, anti-sense or missense HCCR-1 ODN obtained in Example 18, and a growth medium(RPMI-1640) containing 100 nM of HCCR-1 ODN was replaced

every other day. Cells in triplicate dishes were detached and viable cells were counted every other day using trypan blue dye exclusion.

As shown in Fig. 27, until 1 day of treatment, there were no discernable differences in cell growth among sense(□), missense(□) or anti-sense(o) HCCR-1 ODN-treated carcinoma cells. However, after 3 days of HCCR-1 ODN treatment, anti-sense HCCR-1 ODN inhibited lung carcinoma cell growth in a time-dependent manner.

After 7 days exposure to antisense HCCR-1 ODN, the extent of growth inhibition was about 100% for H-358 lung carcinoma cells, while cells exposed to sense or missense HCCR-1 ODN showed growth patterns similar to that of untreated wild-type H-358 cells(control cells,).

Example 20: HCCR-1 gene as a regulator of embryonic kidney development

Because the acquisition of epithelial properties by the fibroblast-derived HCCR-1 cells mimics the mesenchymal to epithelial conversion of cells during the organogenesis of the kidney (Giordano, S. *et al., Proc. Natl. Acad. Sci. USA* **90**, 649-653 (1993): Tsarfaty, I., *et al.*, *Science* **263**, 98-101 (1994)), an experiment was conducted to examine whether HCCR-1 is expressed in a developing kidney.

Total proteins in tissue extracts of fetal 16-, 18- and 20-day rat kidneys, postnatal 1-, 7- and 14-day rat kidneys and adult rat kidney were subjected to SDS-PAGE. HCCR-1 protein in HCCR-1 positive bands were detected by ECL-Western blot detection kit employing rat polyclonal anti-HCCR-1 serum as in Example 14.

The result in Fig. 28 demonstrates that HCCR-1 protein having a relative molecular mass of approximately 40,000 (M_r ~40K) begins to be

overexpressed at fetal 18-day remains at a high expression level up to postnatal 14-day, and decreases to a very low level in adult rat kidney. In Fig. 28, *F* and *P* denote fetal and postnatal, respectively.

A 20-day-old fetal rat kidney was subjected to an immunohistochemical staining as in Example 15. As revealed in Fig. 29 which shows a stained section of the rat kidney(Magnification, × 42), HCCR-1 antibody stained the collecting ducts only (medulla on the left side), which are derived from the ureteric bud (Saxen, L. *Organogenesis of the kidney.* 88-128 (Cambridge University Press, Cambridge, United Kingdom, 1987); Coles, H. S., et al., *Development* **118**, 777-784 (1993)). The developing nephrons in the cortex were not stained (nephrogenic zone on the right side).

Further, a 18-day-old fetal rat kidney was subjected to an immunohistochemical staining as in Example 15 and, then, observed under a differential-interference contrast microscope. As shown in Fig. 30, the basolateral plasma membranes of medullary collecting duct were especially reactive to HCCR-1 antibody(Magnification, × 220).

Because nephrogenesis is stimulated by a distinct ureteric signal, diffusion-limited basolateral molecules (Barasch, J., et al., *Am. J. Physiol.* **271**, F50-F61 (1996)), which trigger mesenchymal to epithelial conversion, it is presumed that the HCCR-1 product may be a mesenchyme-derived regulatory factor (Barasch, J. *et al., Cell* **99**, 377-386 (1999) : Barasch, J. *et al., J. Clin. Invest.* **103**, 1299-1307 (1999)) that stimulates morphogenesis of epithelia in the kidney developmental process and mediates interactions between mesenchyme and epithelia during neoplastic transformation.

The present specification includes the appended Sequencing Listing of 47 nucleic acid or amino acid sequences. Articles of the patent and

scientific periodical literature cited herein are thereby incorporated in their entity by such citation.

While the embodiments of the subject invention have been described and illustrated, it is obvious that various changes and modifications can be made therein without departing from the spirit of the present invention which should be limited only by the scope of the appended claims.